The immune response attenuates growth and nutrient storage in *Drosophila* by reducing insulin signaling

Justin R. DiAngelo[a], Michelle L. Bland[a], Shelly Bambina[b], Sara Cherry[b,1], and Morris J. Birnbaum[a,1]

[a]The Institute for Diabetes, Obesity and Metabolism and [b]Department of Microbiology and Penn Genome Frontiers Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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Innate immunity is the primary and most ancient defense against infection. Although critical to survival, coordinating protection against a foreign organism is energetically costly, creating the need to reallocate substrates from nonessential functions, such as growth and nutrient storage. However, the mechanism by which infection or inflammation leads to a reduction in energy utilization by these dispensable processes is not well understood. Here, we demonstrate that activation of the Toll signaling pathway selectively in the fat body, the major immune and lipid storage organ of the fruit fly, *Drosophila melanogaster*, leads to both induction of immunity and reallocation of resources. Toll signaling in the fat body suppresses insulin signaling both within these cells and non-autonomously throughout the organism, leading to a decrease in both nutrient stores and growth. These data suggest that communication between these two regulatory systems evolved as a means to divert energy in times of need from organismal growth to the acute requirement of combating infection.

fat body | immunity | Toll | insulin

The innate immune system, which provides the most primitive and first line of defense against invading pathogens, recognizes microorganisms via a limited number of pattern recognition receptors. The detection of foreign organisms induces a battery of responses including the production of high concentrations of antimicrobial effectors (1). Therefore, mounting an immune response is an energetically costly process that requires a shift in energy away from nonessential functions (2, 3). During a bacterial infection, this reallocation is evident as nutrient storage is inhibited and adipose tissue triglycerides are hydrolyzed and released (4, 5). Perhaps the most dramatic example of this process is sepsis, when infection leads to a massive, maladaptive mobilization of energy stores that can be life threatening (6).

A pathological state that has recently been postulated to be a disease of nutrient allocation is type 2 diabetes mellitus (T2DM). T2DM results from the inability of insulin to promote glucose uptake into skeletal muscle and adipose tissue and to inhibit gluconeogenesis in the liver during times of nutrient excess, a process known as insulin resistance. While insulin resistance is generally associated with obesity, the primary mechanism linking adipose tissue to insulin responsiveness in other tissues remains unclear. Various hypotheses to explain this interaction include the production of diabetogenic adipokines, the accumulation of intracellular lipid, ER stress, reactive oxygen, and activation of the innate inflammatory response. Initiation of inflammation in animal models and, to some extent, in humans has provided evidence to support this latter model (7–12). Inflammatory stimuli activate at least two highly conserved signaling pathways involving c-Jun NH2-terminal kinase (JNK) and NF-κB that can lead to phosphorylation and inhibition of the insulin receptor substrate family of signaling mediators (7, 9). However, the etiology of the interaction between inflammatory and insulin signaling is still poorly understood.

The fruit fly has often been used as a genetically tractable model system for the study of innate immunity, organismal growth, and energy utilization. *Drosophila* relies exclusively on the innate system, lacking adaptive immunity (13). Depending on the pathogen, infection of the fly initiates one of two distinct signaling cascades, Toll or Imd, and these pathways are remarkably conserved with those in mammals (13, 14). *Drosophila* clears infection through a complex inflammatory-like response involving both cellular and humoral defenses. The fat body is the central organ responsible for the humoral response, synthesizing and secreting antimicrobial peptides into the hemolymph (13). In addition to its essential role as an effector for the immune system, the insect fat body also serves as the primary organ for storage of neutral lipids. Given that this tissue is responsible for these two functions, we posited that the fat body integrates pathogenic and metabolic inputs to adjust the net energy balance in response to infection. To test this hypothesis, we asked whether initiation of innate inflammatory signaling diverts energy away from anabolic processes by interfering with the insulin signaling pathway, a phylogenetically conserved sensor of nutritional abundance whose activation promotes cell and organ growth, development, and nutrient storage (15–18). In this study, we find that activating the Toll pathway inhibits insulin signaling activity resulting in decreased triglyceride storage and larval growth. We propose that this interaction between Toll and insulin signaling has evolved as a means to allocate energy during infection and may reveal the origins of the interaction between inflammation and insulin resistance found in many metabolic diseases.

Results

Activating the Toll, But Not the Imd Pathway, Antagonizes Insulin Signaling. Depending on the microbe, infection of the fly typically leads to the initiation of either the Toll or Imd pathway, which ultimately activates the downstream NF-κB transcription factors dorsal and Dif, or Relish, respectively, leading to transcriptional activation of antimicrobial peptide genes (13). To simulate the innate immune response to pathogens, we used the Gal4/UAS system to express members of the Toll or Imd pathways during larval development, a time when the animals undergo substantial growth and nutrient storage, two insulin-dependent processes (16, 18). To activate the Toll pathway, we expressed a constitutively active form of the Toll receptor, Toll^{10b}, and to activate the Imd pathway, we expressed a constitutively active form of the NF-κB transcription factor RelD (19), using r4-Gal4, a fat body specific driver that is active throughout larval development (20). As expected, expression of Toll^{10b} or RelD induced transcription of their target antimicrobial peptide genes, *Drosomycin* or

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[1]To whom correspondence may be addressed. E-mail: birnbaum@mail.med.upenn.edu and cherrys@mail.med.upenn.edu.

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Diptericin, respectively (Fig. S1). Additionally, we found that Toll, but not Imd, activation reduced triglyceride levels. This contrasts with the increase in triglycerides that is observed when a constitutively active insulin receptor is expressed in the fat body (Fig. 1*A*). Since insulin promotes anabolic metabolism, particularly the synthesis and accumulation of readily accessible energy sources such as triglycerides and glycogen (18), these data led us to test whether the Toll pathway interferes with insulin signaling.

To address this question, we analyzed the phosphorylation status of one of the major kinases in the insulin signaling pathway, dAkt/dPKB (21). In this experiment, we modulated inflammatory signaling exclusively in the female adult fat body using yolk-Gal4, a driver that expresses in the fat body after eclosion, to avoid developmental effects of Toll activation (22). Inducing the Toll pathway by expression of either Toll^{10b} or its downstream adaptor dMyD88 decreased dAkt phosphorylation in the adult female abdominal fat body (Fig. 1*B*). Forced expression of the downstream NF-κB transcription factor Dif also decreased dAkt phosphorylation in the fat body, indicating that the suppression of insulin signaling by Toll is likely transmitted through transcriptional intermediates (Fig. 1*B*). This attenuation of insulin signaling is specific for the Toll pathway as fat-body-specific expression of the Imd pathway receptor, peptidoglycan recognition protein LCa (PGRP-LCa) (23), or RelD, had no effect on dAkt phosphorylation (Fig. 1*C*), despite stimulation of *Diptericin* expression (Fig. S1). One target of the Imd pathway is the stress-activated serine/threonine kinase, Jun kinase (24). In both *Drosophila* and mammals, JNK is a potent antagonist of insulin signaling and has been linked to insulin-resistant states in rodents and man (10, 25). Expression of PGRP-LCa in the *Drosophila* fat body did indeed activate JNK as measured by increased phosphorylation (Fig. S2), but there was no concomitant decrease in phospho-dAkt (Fig. 1*C*). Additionally, the phosphorylation state of JNK was unchanged when the Toll pathway was activated by Toll^{10b} expression, suggesting that JNK activity is not involved in the interaction of the Toll and insulin pathways (Fig. S2). These data suggest that signaling through the Toll but not the Imd pathway antagonizes insulin signaling in the *Drosophila* fat body.

Activating the Toll Pathway by Infection Attenuates Insulin Signaling. In *Drosophila*, infection by Gram-positive bacteria or fungi predominately activates the Toll pathway, while infection by Gram-negative bacteria predominantly activates the Imd pathway (13). To explore whether the suppression of insulin signaling by Toll activation represents a physiological response to infection, we challenged adult flies, a stage in development where the immune response is well characterized, with *Micrococcus luteus*, a Gram-positive bacteria that activates the Toll pathway (26) (Fig. S3). Infection attenuated insulin signaling as measured by a decrease in phospho-dAkt in the fat body 10 h postinfection (Fig. 2*A*). This infection-induced reduction in dAkt phosphorylation was dependent on signaling through the Toll pathway as the decrease in phospho-dAkt was suppressed in animals homozygous for a loss of function mutation in the Toll adaptor protein, *dMyD88* [*EP(2)2133* (27)] (Fig. 2*A*). Importantly, physiological activation of Toll signaling by infection also decreased triglyceride levels, and this phenotype was also suppressed in *dMyD88* mutants, although in these flies the data were much more variable (Fig. 2*B*). Infection of wild-type adult flies with the fungus *Beauvaria bassiana* activated the Toll pathway, as expected (26) (Fig. S3). Fungal infection also decreased phospho-dAkt as well as triglycerides (Fig. 2 *C* and *D*). Adult flies deficient in *dMyD88* did not survive the fungal infection, prohibiting analysis of phospho-dAkt and triglyceride in these animals (27).

To assess the role of physiological activation of the Imd pathway in inhibiting insulin signaling, we infected adult flies with *Escherichia coli*, a Gram-negative bacteria that predominantly induces the Imd pathway but also induces the Toll

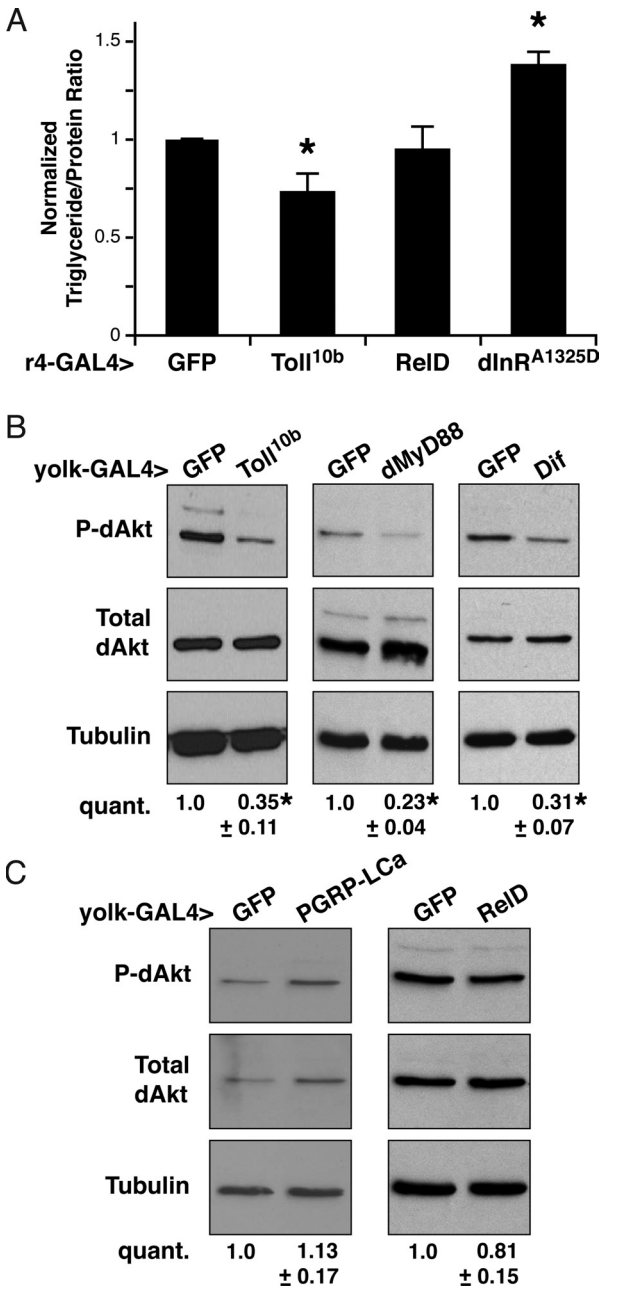

Fig. 1. Activating the Toll, but not the Imd pathway attenuates insulin signaling. (*A*) Triglyceride/protein ratios of *r4-Gal4>Toll^{10b}*, *r4-Gal4>RelD*, and *r4-Gal4>dInR^{A1325D}* whole third instar larvae normalized to *r4-Gal4>GFP* control animals. Each experiment was performed at least three times and values represent the mean ± SEM. *, $P < 0.05$ compared to GFP, unpaired Student's *t*-test. (*B* and *C*) Immunoblot analyses of phospho-dAkt, total dAkt, and tubulin in fat bodies isolated from 4- to 5-day-old adult females of the following genotypes: (*B*) *yolk-Gal4>GFP*, *yolk-Gal4>Toll^{10b}*, *yolk-Gal4>dMyD88*, and *yolk-Gal4>Dif*; (*C*) *yolk-Gal4>GFP*, *yolk-Gal4>PGRP-LCa*, and *yolk-Gal4>RelD*. Each experiment was performed at least three or four times and representative blots are shown. Phospho-dAkt/total dAkt ratios from the *yolk-Gal4>GFP* flies from each experiment were set to 1 and each of the experimental genotypes in (*B*) and (*C*) was normalized to their respective GFP control. Quantification is shown for three independent experiments. Values represent mean ± SEM. *, $P < 0.05$ compared to GFP, unpaired Student's *t*-test.

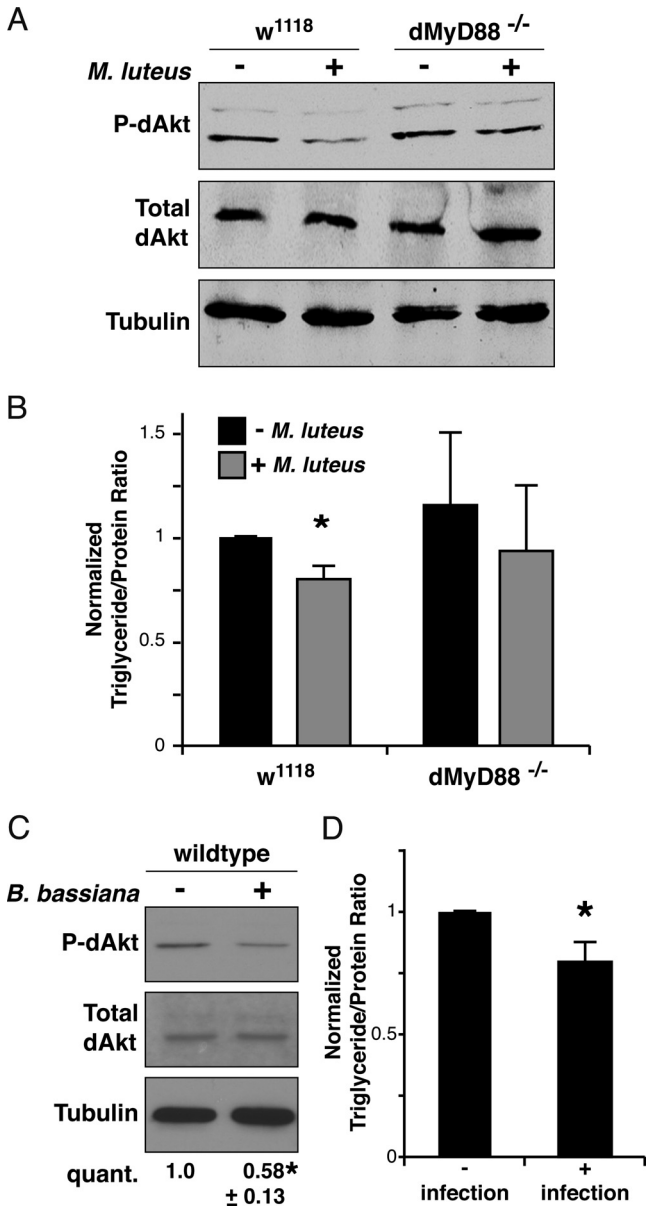

Fig. 2. Insulin signaling is blunted in response to infection-induced Toll activation. (*A* and *B*) Wild-type (w^{1118}) and $dMyD88^{-/-}$ (w^{1118}; $dMyD88^{EP(2)2133}$) adult females (4 to 7 days old) were injected with insulin alone (−) or insulin and *M. luteus* (+) and analyzed 10 h postinfection. (*A*) Immunoblot analyses of phospho-dAkt, total dAkt and tubulin in fat bodies from uninfected (-) and *M. luteus* infected (+) flies. This experiment was performed five times and representative blots are shown. (*B*) Triglyceride/protein ratios were measured and normalized to w^{1118} uninfected controls. Values represent the mean ± SEM. (*C* and *D*) Wild-type (*yw*) adult females (4 to 7 days old) were injected with insulin alone (-) or insulin and *B. bassiana* fungal spores (+) and analyzed 24 h later. (*C*) Immunoblot analyses of phospho-dAkt, total dAkt, and tubulin in fat bodies from uninfected (−) and *B. bassiana*-infected (+) flies. This experiment was performed five times, and a representative blot is shown. Phospho-dAkt/total dAkt ratios from uninfected flies were set to 1 and infected values were normalized to these controls. Quantification is shown for three independent experiments. (*D*) Triglyceride/protein ratios were measured and normalized to uninfected controls. Values represent the mean ± SEM. *, $P < 0.05$ by an unpaired Student's *t*-test.

pathway (26) (Fig. S4 *A* and *B*). While *E. coli* infection had no effect on triglyceride levels, it did result in decreased phosphorylation of dAkt (Fig. S4 *C* and *D*). However, this effect was dependent on the presence of a functional Toll pathway, as it was

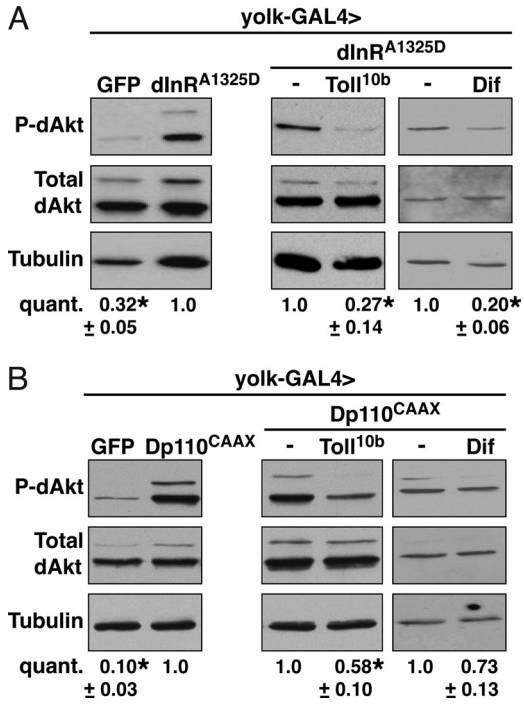

Fig. 3. Toll signaling inhibits insulin signaling downstream of PI3K. (*A* and *B*) Immunoblot analyses of phospho-dAkt, total dAkt, and tubulin in fat bodies isolated from 4- to 5-day-old adult females of the following genotypes: (*A*) *yolk-Gal4>GFP*, *yolk-Gal4>*$dInR^{A1325D}$, *yolk-Gal4>*$dInR^{A1325D}$, $Toll^{10b}$, and *yolk-Gal4>*$dInR^{A1325D}$, *Dif*; (*B*) *yolk-Gal4>GFP*, *yolk-Gal4>*$Dp110^{CAAX}$, *yolk-Gal4>*$Dp110^{CAAX}$, $Toll^{10b}$, and *yolk-Gal4>*$Dp110^{CAAX}$, *Dif*. Each set of experiments was performed at least three times, and representative blots are shown. Phospho-dAkt/total dAkt ratios from the *yolk-Gal4>* $dInR^{A1325D}$ (*A*) or *yolk-Gal4>*$Dp110^{CAAX}$ (*B*) flies from each experiment were set to 1 and each of the other genotypes was normalized to their respective $dInR^{A1325D}$ (*A*) or $Dp110^{CAAX}$ (*B*) control. Quantification is shown for three independent experiments. Values represent the mean ± SEM. *, $P < 0.05$ by an unpaired Student's *t*-test.

suppressed in *dMyD88* mutants (Fig. S4*D*). Together, these data indicate that in *Drosophila* activation of Toll either by genetic manipulation of the signaling pathway or infection decreases insulin signaling in the fat body.

The Toll Pathway Interacts with Insulin Signaling at or Downstream of PI3K. To identify the step in the insulin signaling cascade that is inhibited by Toll signaling, we performed epistasis analysis. To this end, we activated genetically distinct intermediates in the insulin pathway in the adult fat body using yolk-Gal4 and assessed whether Toll could attenuate dAkt phosphorylation. As expected, expression of an active form of the most upstream component of the pathway, the insulin receptor ($dInR^{A1325D}$), increased dAkt phosphorylation (Fig. 3*A*, left panel). However, co-expression of $dInR^{A1325D}$ with $Toll^{10b}$ or Dif reduced dAkt phosphorylation relative to $dInR^{A1325D}$ alone (Fig. 3*A*). These data show that Toll antagonizes insulin signaling downstream of the receptor and argue against a general reduction in the level of circulating insulin-like peptides in flies with activated Toll.

Unlike mammals, *Drosophila* uses not only an insulin receptor substrate (IRS) orthologue (chico), but also the cleaved carboxyl-terminus of the insulin receptor as receptor substrates and scaffolds for the assembly of a functional signaling complex (28, 29). Nonetheless, as in vertebrates, the physiologically relevant consequence of insulin receptor activation is docking and activation of Class I phosphatidylinositide 3-kinase (PI3K). To test where Toll repressed insulin signaling relative to PI3K,

we expressed a constitutively active form of the catalytic subunit of PI3K ($Dp110^{CAAX}$) in the fat body. As observed with the active form of the insulin receptor, $Dp110^{CAAX}$ expression alone increased dAkt phosphorylation. We found that $Toll^{10b}$ or Dif co-expression with $Dp110^{CAAX}$ partially suppressed dAkt phosphorylation (Fig. 3*B*). These data suggest that Toll signaling acts at or downstream of PI3K to antagonize insulin action.

To further assess signaling through the PI3K/Akt pathway, we monitored the subcellular localization of a direct target of dAkt, the transcription factor dFoxo, in the larval fat body as has been previously described (25). In the absence of insulin signaling, dFoxo is localized to the nucleus. Activated dAkt phosphorylates dFoxo leading to its translocation out of the nucleus and into the cytoplasm (30, 31). In larval fat bodies expressing GFP or $Toll^{10b}$ using r4-Gal4, dFoxo exhibited a predominantly nuclear distribution. When $dInR^{A1325D}$ was expressed in the larval fat body, we observed cytoplasmic dFoxo, consistent with the effect on both signaling and dAkt phosphorylation in adult fat bodies. However, when $Toll^{10b}$ was co-expressed with $dInR^{A1325D}$, dFoxo was located in the nucleus (Fig. 4 *A* and *B*). Again, these data support a role for immune signaling in the control of insulin signaling downstream of the receptor.

Fat Body Toll Signaling Non-Autonomously Controls Organismal Growth. The data presented thus far suggest that genetic or pathogenic activation of Toll signaling in the fat body leads to a reduction in triglyceride content through suppression of the insulin signaling cascade. If this reduction in lipid stores truly represents part of a generalized reallocation of resources during times of stress, one would expect that in a developing animal there would be a concomitant arrest in growth, the most energetically "expensive" process for an immature organism. However, since infection stimulates Toll signaling primarily in the fat body, a reduction in organismal growth would likely occur through a non-autonomous mechanism. To test this hypothesis, we first asked whether activation of the Toll pathway in the larval fat body influences growth and developmental timing. Strikingly, expression of $Toll^{10b}$ in the larval fat body using r4-Gal4 led to an overall reduction in body size (Fig. 5*A*). Moreover, these animals were developmentally delayed by approximately 24–36 h and had reduced viability. However, some flies did survive to adulthood and were also smaller than wild-type animals, phenocopying hypomorphic insulin-signaling pathway mutants (Fig. 5*B*) (16). Given the resemblance of fat body-specific Toll activation to a global reduction in insulin signaling, we measured the activation state of dAkt in the larvae. Immunoblot analysis revealed a decrease in phospho-dAkt levels in whole larval extracts, to which fat body protein contributes only about 11%, indicating that the diminution in growth was most likely due to a generalized attenuation in insulin signaling (Fig. 5*C*).

These data suggest two models to explain the non-autonomous effect of Toll signaling in the fat body on larval growth. First, activation of Toll might directly produce an extracellular factor that is secreted from the fat body and transported to the rest of the organism inhibiting growth; alternatively, the global decrease in growth could be secondary to the reduction in larval insulin signaling in the fat body. To distinguish between these models, we tested whether the small larval size could be suppressed by the simultaneous fat body expression of an active form of dAkt (myrAkt) with $Toll^{10b}$, thus bypassing the block in insulin signaling. Indeed, co-expression of $Toll^{10b}$ and myrAkt in the larval fat body restored third instar larvae (Fig. 5*A*) and adults (Fig. 5*B*) to approximately wild-type size. This phenotype is not due to additive effects of myrAkt expression, as myrAkt alone had no effect on larval or adult size (Fig. 5 *A* and *B*). Since Toll activity is restricted to the fat body, these data argue that it is the cell-autonomous reduction in fat body insulin signaling that leads to the non-autonomous attenuation in organismal growth (Fig. 5*D*).

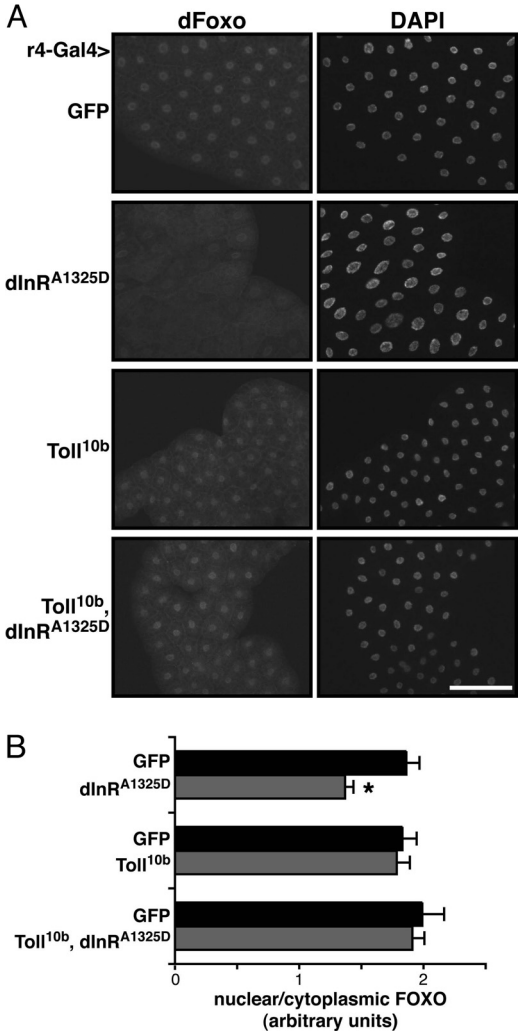

Fig. 4. Subcellular distribution of dFoxo correlates with activity of the insulin signaling pathway. (*A*) dFoxo (left) and DAPI (right) staining of fat bodies dissected from third instar larvae of the following genotypes: *r4-Gal4>GFP* (top), *r4-Gal4>dInR^{A1325D}*, *r4-Gal4>Toll^{10b}*, and *r4-Gal4>Toll^{10b}*, *dInR^{A1325D}* (bottom). (Scale bar, 100 μm.) (*B*) The ratio of nuclear to cytoplasmic dFoxo was quantified using Image J. Each experimental group (gray bar) is shown compared to its respective *r4-Gal4>GFP* control (black bar). n = 9–11 fat bodies (GFP), 11 ($dInR^{A1325D}$), 7 ($Toll^{10b}$), and 20 ($Toll^{10b}$, $dInR^{A1325D}$), Values represent mean ± SEM. *, $P < 0.05$ vs. control by an unpaired Student's *t*-test.

Discussion

Two of the most primitive, phylogenetically conserved metazoan regulatory systems are innate immunity and insulin action, which control the response to pathogens and anabolic processes such as nutrient storage, reproduction and growth, respectively. In this study, we demonstrate a fundamental interaction between these pathways, in which activating innate immunity brings about a reduction in insulin signaling locally leading to systemic growth impairment. This path of communication may have evolved as a mechanism to reallocate energy utilization from non-essential processes to the more immediate need of combating infection. For example, *Drosophila* mutants that abrogate nutrient signaling such as the fly IRS homolog *chico*, have improved survival after infection and this is not due to increased expression of antimicrobial peptides (32). While the mechanism underlying

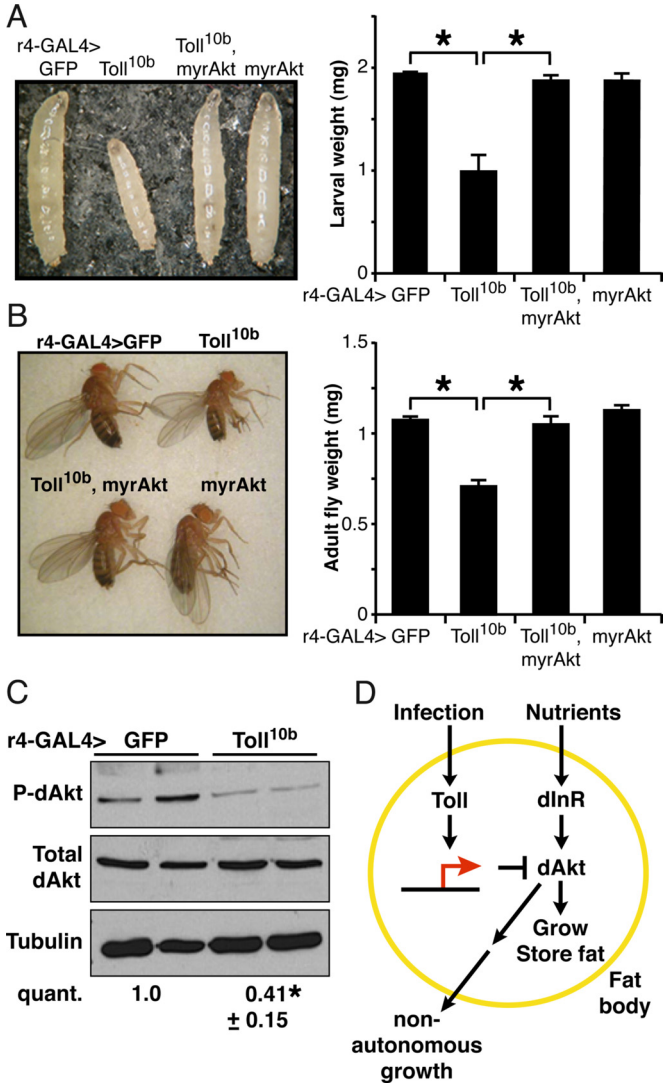

Fig. 5. Toll signaling in the fat body nonautonomously inhibits organismal growth. (*A* and *B*) Images (left panels) and body weights (right panels) of (*A*) wandering third instar larvae and (*B*) 1- to 2-day-old adult females of the following genotypes: *r4-Gal4>GFP*, *r4-Gal4>Toll^{10b}*, *r4-Gal4>Toll^{10b}*, *myrAkt*, and *r4-Gal4>myrAkt*. Values are the mean ± SEM. *, $P < 0.01$ by an unpaired Student's *t*-test. (*C*) Immunoblot analyses of phospho-dAkt, total dAkt and tubulin in protein extracts from whole *r4-Gal4>GFP* and *r4-Gal4>Toll^{10b}* third instar larvae. This experiment was performed at least three times, and a representative blot is shown. Phospho-dAkt/total dAkt ratios from the *r4-Gal4>GFP* flies from each experiment were set to 1 and the *r4>Toll^{10b}* genotype was normalized to the GFP control. Quantification is shown for three independent experiments. Values are the mean ± SEM. *, $P < 0.05$ by an unpaired Student's *t*-test. (*D*) A model illustrating the interaction between fat body Toll and insulin signaling in response to infection to regulate nutrient storage locally and animal growth nonautonomously.

the improved survival of infected *chico* mutants is unclear, our data support a model whereby these animals are resistant to infection due to decreased insulin signaling, which reallocates energy utilization to immune control from energetically costly processes such as growth and reproduction, as evidenced by the fact that these mutants are small and have reduced fecundity (28, 33). Likewise, chronic activation of immune signaling in the fly decreases reproductive fitness and longevity, although how these processes are linked molecularly is unknown (34, 35). Our study suggests that these phenotypes are mediated by infection-induced inhibition of dAkt signaling.

How the interaction between immune and metabolic signals is coordinated is unknown. We find that signaling through Toll, but not Imd, decreases insulin pathway activity. However, why only one of the major immune signaling cascades interacts with insulin signaling is unclear. One possibility is that this crosstalk first arose to function in another context. For example, both the Toll and insulin pathways have important roles in development, while the Imd pathway functions solely in immunity (16, 22, 36). Another possibility is that the Toll pathway is activated in response to many if not all pathogens including those once thought to be exclusively restricted to the Imd pathway such as *E. coli*. This cross-activation of these pathways is evident in microarray studies performed on fungus-infected flies (37) and the fact that challenge with *E. coli* leads to *Drosomycin* induction which is dependent upon Toll signaling (Fig. S4*A*). Therefore, while the Toll but not Imd pathway intersects with the insulin signaling pathway, it may be that a broad spectrum of microbial challenges display this cross-activation and explains the Toll pathway-dependent decrease in phospho-dAkt after Gram-negative bacterial infection observed in this study (Fig. S4*D*).

The Toll pathway appears to activate transcription to antagonize insulin signaling, as overexpression of the NF-κB family member Dif is sufficient to decrease dAkt phosphorylation both basally and in a dInR^{A1325D} background. Previous RNA expression profiling has identified a number of genes regulated by the Toll pathway, but none are obvious modulators of insulin action (37). Interestingly, one of the largest groups of genes regulated by Toll are proteases (37), raising the possibility that a protein important for promoting insulin signaling may be degraded in response to Toll activation. Additionally, these experiments revealed that expression of lipases is increased in response to fungal infection (37), which is consistent with our data that triglycerides were decreased after exposure to fungus. Lipid metabolic genes are also dysregulated in flies infected with the tuberculosis-like pathogen, *Mycobacterium marinum*, by decreasing insulin signaling activity, but the immune pathway mediating these effects is unknown (38).

The fat body is a nutrient storage and immune organ of the fly, and as such, integrates metabolic and inflammatory signals to coordinate energy use. While nutrient metabolism and innate immunity take place in a single organ in the fly, in mammals these processes reside in adipocytes and macrophages, respectively, two seemingly disparate cell types that in fact display similar gene expression patterns and overlapping functional properties (39). Thus, it is likely that in mammals, multiple related cell types participate in processes that recapitulate the workings of the *Drosophila* fat body, using signals and pathways that are fundamentally analogous. For example, infection in rodents induces adipose tissue lipolysis, which provides fatty acids for the production of energy and synthesis of membranes by inflammatory cells (3).

Lastly, these studies have implications for understanding the evolutionary origins of some of the most prevalent human maladies in Western societies, such as the metabolic syndrome, T2DM, and polycystic ovarian syndrome. These three diseases all stem from insulin resistance, which has been proposed to be a pathological response to innate inflammation (39). The demonstration here of Toll-induced suppression of insulin signaling in *Drosophila* as an effective means to divert energy from growth to inflammation provides an evolutionary framework to understand the corresponding maladaptive process in mammals, in which a state of over-nutrition obviates the need for substrate reallocation but leads to numerous metabolic complications. Moreover, the identification of a conserved regulatory system in a genetically-tractable organism provides an approach for the elucidation of the relevant molecular interactions.

Materials and Methods

Fly Strains. Flies were grown on standard cornmeal dextrose medium supplemented with dry yeast. Larval crosses were performed at 18–20 °C and adult crosses were performed at 25 °C. Control animals were cultured in the same vials as experimental animals to account for larval crowding. The following fly strains were used in this study: yolk-Gal4 (40), r4-Gal4 (20), UAS-Toll^{10b} (41), UAS-dMyD88 (27), *w^{1118}*; *MyD88 $^{EP\ (2)2133}$* (27), UAS-Dif (a gift from Tony Ip), UAS-PGRP-LCa (23), UAS-Dp110^{CAAX} (42), UAS-dInR^{A1325D}, and UAS-GFP and *w^{1118}* (Bloomington). The UAS-Toll^{10b}, UAS-dInR^{A1325D} flies were generated by recombination. The UAS-RelD line was made by cloning the RelD construct described in Han and Ip (19) into pUAST and performing P-element transformation by standard procedures.

Immunocytochemistry. Fat bodies were dissected from larvae 96 h after egg lay, fixed in 4% paraformaldehyde for 20–45 min, blocked in 10% normal donkey serum (Jackson ImmunoResearch) in PBS with 0.1% Triton X-100 (NDS-T) for 30 min, then incubated overnight at 4 °C with rabbit anti-dFOXO (31) diluted 1:1,500 in 1% NDS-T. The next day, fat bodies were washed in 1% NDS-T, incubated with donkey anti-mouse Cy3 (Jackson ImmunoResearch) secondary antibody for 2 h at room temperature, counterstained with DAPI, and mounted on slides in Fluoromount G (Electron Microscopy Sciences). Images were collected on a Nikon Eclipse E800 microscope at 20× magnification using MetaMorph software.

Infection Experiments. For septic injury, wild-type adult females aged 4–7 days were challenged with a suspension of *Beauvaria bassiana* spores, *Micrococcus luteus*, or *E. coli* (26) in 1 mg/mL insulin in PBS at room temperature as previously described (43). Uninfected control animals were injected with insulin alone.

More detailed materials and methods are found in the *SI Text*.

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